

TREATMENT OF DEMYELINATING AUTOIMMUNE DISEASE WITH MODIFIED ORDERED PEPTIDES

GOVERNMENT SUPPORT

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BACKGROUND OF THE INVENTION

10 *Introduction*

 Multiple sclerosis (MS) is an acquired, inflammatory, demyelinating disease of the central nervous system (CNS). In MS, cells of the immune system invade and destroy myelin, the fatty material that insulates nerves in the brain and spinal cord; other CNS cells produce a hardened sclerotic lesion (plaque)
15 around the multiple demyelinated sites. Neurologic findings suggest lesions in separate areas of the CNS that have occurred at different times.

 Multiple sclerosis (MS) is the most common autoimmune disease involving the nervous system. In the United States approximately 400,000 individuals suffer from MS. The cause of the disease is unknown, but genetic factors are
20 important. The concordance rate among monozygotic twins is 30%, a 10-fold increase over dizygotic twins or first-degree relatives. The higher incidence rate among monozygotic twins emphasizes the importance of genetic factors, but the discordance rate of 70% among identical twins illuminates the role of nongenetic factors on disease penetrance. Among genetic factors, HLA class II genes exert
25 an influence, with HLA DR2 carrying a 4-fold relative risk for northern European caucasoids.

 A typical presentation of MS involves an initial course, running for several years to more than a decade, manifest by episodes of relapse followed by remission. Relapses often follow an episode of a viral infection of the upper
30 respiratory system or gastrointestinal tract. In about one half of MS cases the disease progresses to a more chronic phase. Clinical problems may include disturbances in visual acuity, sometimes culminating in blindness; double vision; motor disturbances affecting walking and use of the hands; incoordination; bowel and bladder incontinence; spasticity; and sensory disturbances including loss of

touch, pain, and temperature and proprioception. The pathology of the disease lies entirely in the central nervous system and is characterized by a classic picture of inflammation surrounding venules and extending into the myelin sheath.

5 Immune responses to various components of the myelin sheath have been detected in MS patients, including myelin basic protein (MBP), proteolipid protein (PLP), transaldolase, and 2',3' cyclic nucleotide 3'phosphodiesterases (CNP), as well as two members of the immunoglobulin supergene family found in the myelin sheath, myelin oligodendroglial glycoprotein (MOG) and myelin-associated
10 glycoprotein (MAG) (Steinman *et al.* (1995) Mol. Med. Today 1:79-83). In addition, some inducible heat shock proteins, including crystallin-B, can be detected in glial cells in MS lesions and can stimulate an immune response in MS patients.

In human MS patients the following myelin proteins and epitopes were
15 identified as targets of the autoimmune T and B cell response. Antibody eluted from MS brain plaques recognized myelin basic protein (MBP) peptide 83-97 (Wucherpfennig *et al.*, *J Clin Invest* 100:1114-1122, 1997). Another study found approximately 50% of MS patients having peripheral blood lymphocyte (PBL) T cell reactivity against myelin oligodendrocyte glycoprotein (MOG) (6-10%
20 control), 20% reactive against MBP (8-12% control), 8% reactive against PLP (0% control), 0% reactive MAG (0% control). In this study 7 of 10 MOG reactive patients had T cell proliferative responses focused on one of 3 peptide epitopes, including MOG 1-22, MOG 34-56, MOG 64-96 (Kerlero de Rosbo *et al.*, *Eur J Immunol* 27, 3059-69, 1997). T and B cell (brain lesion-eluted Ab) response
25 focused on MBP 87-99 (Oksenberg *et al.*, *Nature* 362, 68-70, 1993). In MBP 87-99, the amino acid motif HFFK is a dominant target of both the T and B cell response (Wucherpfennig *et al.*, *J Clin Invest* 100, 1114-22, 1997). Another study observed lymphocyte reactivity against myelin-associated oligodendrocytic basic protein (MOBP), including residues MOBP 21-39 and MOBP 37-60 (Holz *et al.*, *J Immunol* 164, 1103-9, 2000). Using immunogold conjugates of MOG and
30 MBP peptides to stain MS and control brains both MBP and MOG peptides were recognized by MS plaque-bound Abs (Genain and Hauser, *Methods* 10, 420-34, 1996).

Neuropathological findings suggest that antibodies may play a role in lesion formation in some multiple sclerosis patients. (Storch et al. *Ann. Neurol.* 43: 465-71, 1998). Autoantibodies recognizing several myelin proteins including MBP (Sellebjerg et al., *Ann Neurol.* 38: 943-50: 1995), proteolipid protein (*Ibid*),
5 myelin-associated glycoprotein (Baig et al., *Neurology* 41: 581 –7: 1991) and 2',3' – cyclic nucleotide 3' – phosphodiesterase (Walsh and Murray, *JCI* 101: 1923-31: 1998) are present in multiple sclerosis patients but their role in disease pathogenesis is enigmatic and controversial.

10 A key autoimmune response in MS is targeted to certain regions of myelin basic protein. The major T and B cell response in the central nervous system of MS patients who are HLA DR2 (about two thirds of patients) is directed to a region between residues 84 and 103 of MBP (Steinman (1995) *Nature* 375:739-740; Warren et al. (1995) *P.N.A.S.* 92:11061-11065). The B cell response to MBP
15 in MS has also been studied extensively. IgG purified from brain lesions reacted with the same region of MBP, p85-96, that is the immunodominant T cell epitope in MS patients who are HLA DR2b (DRB1*1501) and overlaps with the T cell epitope in MS patients who are DR2a (DRB5*0101).

20 *Relevant Literature*

Copolymer-1 is a mixture of polypeptides composed of alanine, glutamic acid, lysine, and tyrosine in a molar ratio of approximately 6:2:5:1, respectively. It is synthesized by chemically polymerizing the four amino acids forming products with average molecular weights of 23,000 daltons (U.S. Pat. No. 3,849,550). Cop
25 1 binds promiscuously, with high affinity and in a peptide-specific manner to purified MS-associated HLA-DR2 (DRB1*1501) and rheumatoid arthritis-associated HLA-DR1 (DRB1*0101) or HLA-DR4 (DRB1*0401) molecules (Fridkis-Hareli et al. (1999) *J Immunol* 162(8):4697-704). Protruding N-terminal ends of Cop 1 bound to HLA-DR1, -DR2, or -DR4 molecules were then treated
30 with aminopeptidase I, followed by elution, HPLC, and pool sequencing. In contrast to untreated or unbound Cop 1, this material exhibited distinct motifs at some positions with increases in levels of E at the first and second cycles, of K at the second and third cycles, and of Y (presumably at P1 of the bound peptide) at

the third to fifth cycles, regardless of the HLA-DR molecule employed. No preference was seen at the following cycles that were mainly A.

Cop-1 has been recently approved as a treatment for relapsing multiple sclerosis (MS). Evidence demonstrates that Cop-1 induces active suppression of CNS-inflammatory disease in animal models (Aharoni *et al.* (1997) P.N.A.S. 94(20):10821-6). In humans, Copaxone treatment was found to lead to a significant reduction in the mean annual relapse rate and stabilization of disability. The treatment was accompanied by an elevation of serum IL-10 levels, suppression of the pro-inflammatory cytokine TNF alpha mRNA, and an elevation of the anti-inflammatory cytokines TGF-beta and IL-4 mRNAs in PBLs (Miller *et al.* (1998) J Neuroimmunol 92(1-2):113-21).

Treatment of murine experimental autoimmune encephalomyelitis with a myelin basic protein peptide analog is described by Reiseter *et al.* (1998) J Neuroimmunol 91(1-2):156-70. A single administration of the MBP peptide analog, Ac1-11[4Y], reduced disease severity, accompanied by a dramatic and selective loss of neutrophil pleiocytosis. A longer course of peptide therapy resulted in complete recovery from clinical signs of disease, and decreased pleiocytosis by all cell types. Wraith *et al.* (1989) Cell 59:247-255 describe antigen recognition in autoimmune encephalomyelitis and the potential for peptide mediated immunotherapy. Sakai *et al.* (1989) Proceedings of the National Academy of Sciences USA 86:9470-9474 describe the prevention of experimental encephalomyelitis with peptides that block interaction of T cells with major histocompatibility complex proteins. Karin *et al.* (1994) J.E.M. 180:2227-2237 demonstrate the reversal of experimental autoimmune encephalomyelitis by a soluble variant of a myelin basic protein epitope.

It has been reported that administration of myelin basic protein can lead to immune tolerance (see, for example, Steinman *et al.* (1977) Nature 265:173; Tonegawa (1997) J Exp Med 186(4):507-15; Hafler *et al.* (1997) Ann N Y Acad Sci 835:120-31; Kennedy *et al.* (1997) J Immunol 159(2):1036-44). Various forms of Ag-specific tolerance have been demonstrated, included the administration of peptide coupled splenocytes, i.p. administration in incomplete adjuvant, oral and nasal administration.

SUMMARY OF THE INVENTION

Methods and compositions are provided for the treatment of demyelinating autoimmune diseases, including experimental autoimmune encephalomyelitis and multiple sclerosis, by administering to the host two or more therapeutic ordered peptide(s) or one or more substituted therapeutic ordered peptides or combinations of therapeutic ordered peptides and substituted therapeutic ordered peptides. One such MBP therapeutic ordered peptide of this invention comprises the ordered amino acid motif {SEQ ID NO:1} [${}^1E^2Y^3Y^4K$]_n, where n is from 2 to 6, modified at the amino or carboxy terminal end. The ordered motif may start at residue 1, as shown, or may start at a different position, e.g. {SEQ ID NO:2} YYKEYYYKE; {SEQ ID NO:3} YKEYYYKEY; etc. A MOG therapeutic ordered peptide of this invention comprises the ordered amino acid motif (${}^1Y^2R^3E^4Y^5E^6Y^7E$)_n where n is from 2 to 10. The MOG therapeutic ordered peptide of this invention may be modified at the amino or carboxy terminal end. A PLP therapeutic ordered peptide of this invention comprises the ordered amino acid motif (${}^1Y^2G^3K^4E^5L^6G^7E^8Y$)_n where n is from 2 to 10. The PLP therapeutic ordered peptide of this invention may be modified at the amino or carboxy terminal end. Other therapeutic ordered peptides of this invention include such peptides from cyclic nucleotide phosphodiesterase (CNPase), myelin associated glycoprotein (MAG), myelin-associated oligodendrocytic basic protein (MBOP), and alpha-B-crystallin (a heat shock protein). Therapeutic ordered peptides of other proteins and epitopes, identified to be targets of the autoimmune T and B cell responses, can be designed and administered using the teaching of this invention.

The compositions of the present invention may be synthesized by conventional methods known in the art, e.g. expression in a recombinant system, solid phase peptide synthesis, etc. The therapeutic ordered peptides are formulated in a biologically acceptable carrier, and administered by a route to enhance the autoimmune suppressive effects of the treatment. Typically, the therapeutic ordered peptides are administered on a regular basis to patients suffering from multiple sclerosis. In a preferred embodiment, the composition is lyophilized and formed into an aqueous solution suitable for subcutaneous

injection and administered on a regular basis in accordance with the method of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 is a graph showing the prevention of EAE in rats treated with therapeutic ordered peptides. MBP therapeutic ordered peptide {SEQ ID NO:4} EYYKEYYKEYYK prevents the development of EAE in Lewis rats. Animals were injected with an emulsion of 0.1 mg of MBPp85-99 in complete Freund's adjuvant for EAE induction. Ten days later, when the clinical manifestations of disease became apparent, a single intra-peritoneal dose of MBP therapeutic ordered peptide {SEQ ID NO:4} EYYKEYYKEYYK (squares), {SEQ ID NO:5} KYYKYYKYYKYY (triangles), or PBS (circles) was administered. Results are expressed as mean disease score of groups of six animals.

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Figure 2 is a graph depicting the reduction in relapse rates in mice with EAE treated with ordered peptides. Animals were induced for EAE with an emulsion of 0.1 mg of PLPp139-151 in complete Freund's adjuvant (day 0). Mice were randomized into equal groups at the peak of disease and were treated at days 17, 29, and 36 after EAE induction with intravenous, intra-peritoneal or subcutaneous administration of EYYKEYYKEYYK (MBP therapeutic ordered peptide) or Copaxone at two different dosages, 0.5 mg per mouse or 0.05 mg per mouse. Both the MBP therapeutic ordered peptide and Copaxone were dissolved in mannitol. Results are expressed as relapse rates per mouse, and show that the MBP therapeutic ordered peptide and Copaxone reduce relapse rates.

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Figure 3 is a table shows the ordered peptide blocking MHC binding by the native peptide. The ability of MBP therapeutic ordered peptide EYYKEYYKEYYK, substituted MBP therapeutic ordered peptide D-Ala-EYYKEYYKEYYK-amide, or Copaxone to block the binding of the native peptide to either a mouse or rat MHC was measured by FACS analysis. As shown in the table as indicated by the lower mean fluorescence intensity (MFI), the substituted

D-ala form of the therapeutic ordered peptide and Copaxone was more effective in blocking either MHC than the non-D-ala form.

Figure 4 is a graph showing the blocking by the therapeutic ordered peptide of T cell proliferation. The ability of MBP therapeutic ordered peptide (EYYKEYYKEYYK), substituted MBP therapeutic ordered peptide (D-Ala-EYYKEYYKEYYK-amide), or Copaxone to block the proliferation of a PLPp139-151 specific T. cell line was measured in a proliferation assay. As shown in the graph there is a dose dependent reduction in T cell proliferation with the substituted MBP D-Ala form of the therapeutic ordered peptide that exceeds the reduction in T cell proliferation for either the unmodified ordered peptide or for Copaxone.

Figure 5 is a graph showing the blocking of induction of EAE by the substituted D-Ala form of the therapeutic ordered peptide. Lewis rats were injected with an emulsion of 0.1 mg of MBPp85-99 in complete Freund's adjuvant for EAE induction. Either of 0.5 mg MBP therapeutic ordered peptide EYYKEYYKEYYK, substituted MBP therapeutic ordered peptide D-Ala-EYYKEYYKEYYK-amide, or Copaxone was mixed into the encephalitogenic emulsion. Results are expressed as mean disease score of 12-13 animals. The substituted D-Ala therapeutic ordered peptide was more effective in blocking EAE induction than the un-substituted therapeutic ordered peptide.

Figure 6 shows cytokine production from T cells derived from mice immunized with D-ala modified ordered peptide, non-modified ordered peptide, and control peptides including Cop1 and ovalbumin (OVA). OVA is known to cause an induction of Th1 type of T cells after immunization. There is an increased production of IL4 and IL10 in the D-ala-ordered peptide (D-ala-EYYK) immunized T cell lines, but not in the non-modified ordered peptide (EYYK) immunized T cell lines. As controls, Cop1 caused an increase in these two Th2 cytokines as expected, and OVA did not cause an increase in these cytokines also as expected. These data imply that the D-ala modified form of the ordered peptide can cause Th2 induction but that the unmodified peptide cannot.

DETAILED DESCRIPTION OF THE EMBODIMENTS

5 Demyelinating autoimmune diseases, including experimental autoimmune encephalomyelitis and multiple sclerosis, are treated by administering a therapeutic ordered peptide. The therapeutic ordered peptides are formulated in a pharmaceutically acceptable carrier for a convenient route of administration, which may be sub-cutaneous, oral, by inhalation, *etc.* as known in the art.

10 The subject methods are used for prophylactic or therapeutic purposes. As used herein, the term "treating" is used to refer to both prevention of disease, and treatment of pre-existing conditions. The prevention of autoimmune disease is accomplished by administration of the peptide prior to development of overt disease. The treatment of ongoing disease, in order to stabilize or improve the
 15 clinical symptoms of the patient, is of particular interest. Such treatment is desirably performed prior to loss of function in the affected tissues. Evidence of therapeutic effect may be any diminution in the severity of disease, particularly measuring the frequency of relapses in patients being treated with the ordered peptides, which may be the length of time the patient is relapse free, or the mean
 20 relapse frequency.

Therapeutic ordered peptides of the present invention comprise eight to
 eighty amino acids representing a consensus sequence of a protein identified as
 a target of the autoimmune T and B cell response. The myelin proteins MBP,
 MOG, PLP, MAG and cyclic nucleotide phosphodiesterase are examples of
 25 proteins that are the target of the autoimmune response for which a therapeutic
 ordered peptide would be developed according to the teaching of this invention. For example, the MBP therapeutic ordered amino acid motif is {SEQ ID NO:1}
 $[{}^1\text{E}{}^2\text{Y}{}^3\text{Y}{}^4\text{K}]_n$, where n is from 2 to 6. The MBP therapeutic ordered motif may start
 at residue 1, as shown, or may start at a different position, *e.g.* {SEQ ID NO:6}
 30 YYKEYYKEYYKE; {SEQ ID NO: 7} KEYYKEYYKEYY, *etc.* The total length of
 the MBP therapeutic ordered peptide sequence will usually be at least about 8
 amino acids in length and not more than about 24 amino acids in length, usually
 at least about 10 and not more than about 20. Specific MBP therapeutic ordered
 peptides of interest include the sequence {SEQ ID NO:4} EYYKEYYKEYYK. In

the case of the MOG therapeutic ordered peptide the amino acid motif is $[^1Y^2R^3E^4Y^5E^6Y^7E]_n$ where n is from 2 to 10. The MOG therapeutic ordered peptide motif may start at residue 1 or may start at a different position, REYEYEYREYEYEYREYEYE, or EYEYEYREYEYEYREYEYE. The total length
5 of the MOG therapeutic ordered peptide will usually be at least about 14 amino acids in length and not more than 70 amino acids in length, usually about 18 amino acids and not more than about 42. In the case of the PLP therapeutic ordered peptide the amino acid motif is $[^1Y^2G^3K^4E^5L^6G^7E^8Y]_n$ where n is from 2 to 10. The PLP therapeutic ordered peptide motif may start at residue 1 or may
10 start at a different position, e.g. GKELGEYYGKELGEYYGKELGEY, or KELGEYYGKELGEYYGKELGEYYG etc. The total length of the PLP therapeutic ordered peptide sequence will usually be at least about 16 amino acids in length and not more than about 80 amino acids in length, usually at least about 20 and not more than about 48.

15 Included within the scope of therapeutic ordered peptides as that term is used herein are amino acid sequence variants. Such therapeutic ordered peptides are referred to herein as "substituted" or "modified" therapeutic ordered peptide. The amino acid sequence variants of therapeutic ordered peptides fall into two classes; either substitutional or additional. The therapeutic ordered
20 peptide variants may be prepared by site specific mutagenesis of nucleotides in the DNA encoding the therapeutic ordered peptide if a recombinant expression system is used or by altering the synthetic scheme in solid phase peptide synthesis. Amino acid sequence variants are characterized by the predetermined nature of the variation. The therapeutic ordered peptide variants typically exhibit
25 the same qualitative biological activity as the therapeutic ordered peptide, for example MHC binding, effect on T cell proliferation, effect on disease severity and relapse rate in EAE etc. The peptide may consist only of the ordered repeats, or may be extended at either terminus by the addition of other amino acid residues.

30 Modification and changes may be made in the structure of the ordered peptide and still obtain a molecule having the desired characteristic of suppressing demyelinating autoimmune disease. The desired properties may be determined, at least in part, in an *in vitro* assay, where binding to the MHC

antigen HLA-DR, particularly HLA-DR2 (DRB1*1501), is indicative of the relevant biological activity. The modified ordered peptides of this invention are likely to bind MHC antigen HLA-DR2 as well as other MHC antigens as would be known to one of ordinary skill in the art. For example, in addition to binding HLA-DR2 modified PLP ordered peptides may also bind HLA-DR15. Another example, modified MOG ordered peptides in addition to their likely binding of HLA-DR2 may also bind HLA-DRB1, HLA-DRB5, and HLA-DR4.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of function. It will be understood by one of skill in the art that various changes (such as to protein stability or efficiency) may be made in the sequence of the ordered peptide without appreciable loss of their biological utility or activity, particularly as to the addition of terminal amino acids. So long as a change maintains the binding properties and immunological activity, the resultant protein will be considered a biologically functional equivalent for the purposes of the invention.

Amino acid substitutions are typically of single residues: insertions usually will be on the order of about from 1 to 4 amino acid residues; and deletions will range about from 1 to 4 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final substituted therapeutic ordered peptide.

Substitutional variants are those in which at least one residue of a therapeutic ordered peptide has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following table when it is desired to finely modulate the characteristics of a therapeutic ordered peptide.

| Original Residue | Exemplary Substitutions |
|------------------|-------------------------|
| Ala | Ser |
| Arg | Lys |
| Asn | Gln; his |
| Asp | Glu |
| Cys | Ser |
| Gln | Asn |

| | |
|-----|-------------|
| Glu | Asp |
| Gly | Pro |
| His | Asn;gln |
| Ile | Leu;val |
| Leu | Ile;val |
| Lys | Arg;gln;glu |
| Met | Leu;ile |
| Phe | Met;leu;tyr |
| Ser | Thr |
| Thr | Ser |
| Trp | Tyr |
| Tyr | Trp; phe |
| Val | Ile; leu |

Changes in function are made by selecting substitutions that are less conservative than those in the foregoing table, i.e. selecting residues that differ more significantly in their effect on maintaining the structure of the ordered peptide, the charge or hydrophobicity of the ordered peptide, or the bulk of the side chain. Substitutions which in general are expected to produce the greatest changes in the ordered peptide will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

Deletions of cysteine or other labile residues also may be desirable, for example in increasing the oxidative stability of the ordered peptide. Deletions or substitutions of potential proteolysis sites, e.g. Arg Arg, is accomplished by deleting one of the basic residues or substituting one by glutamyl or histidyl residues. The stability of the ordered peptide may be improved by D-amino acid additions or substitutions. D-amino acid additions at the N- and/or C-terminal of the ordered peptide, as well as internal D-amino acid substitutions, are made to

maintain the helical structure of the peptide, and to maintain the biological characteristics of the ordered peptide while improving the stability and half-life of the ordered peptide. Certain D-amino acid additions or substitutions may enhance the biological activity of the therapeutic ordered peptide as described
5 herein.

The therapeutic ordered peptides may be provided in a variety of ways, being joined to non-wild-type flanking regions, as fused proteins, joined by linking groups or directly covalently linked through cysteine (disulfide) or peptide linkages. The therapeutic ordered peptides may be joined to a single amino acid,
10 either a D- or L- amino acid, at the N- or C-terminus or a chain of amino acids. The fused peptides may be extended to provide convenient linking sites, e.g. cysteine or lysine, to enhance stability, to bind to particular receptors, to provide for site-directed action, to provide for ease of purification, to alter the physical characteristics (e.g. solubility, charge, etc.), to stabilize the conformation, etc.
15 The therapeutic ordered peptide may be N-terminal, C-terminal or internal in relation to these added sequences.

The therapeutic ordered peptide may be linked through a variety of bi-functional agents, such as maleimidobenzoic acid, methylthioacetic acid, mercaptobenzoic acid, S-pyridyl dithiopropionate, etc. The oligopeptides may be
20 linked to proteins to provide site-directed action. The oligopeptides may be linked, particularly by an intracellular cleavable linkage, to antibodies for site directed action. For conjugation techniques, see, for example, U.S. Pat. Nos. 3,817,837; 3,853,914; 3,850,752; 3,905,654; 4,156,081; 4,069,105; and 4,043,989, which are incorporated herein by reference. The oligopeptides may
25 also be modified by incorporation into the lumen of vesicles, e.g. liposomes, which in turn may be bound to ligands or receptors for direction to particular cells or tissue.

For therapy, the therapeutic ordered peptides may be administered topically or parenterally, e.g. by injection at a particular site, including
30 subcutaneously, intraperitoneally, intravascularly, or the like or transdermally, as by electrotransport. In a preferred embodiment, subcutaneous injection is used to deliver the therapeutic ordered peptide. The oligopeptides may also be administered in a sustained release formulation or osmotic pump, to provide a depot of active peptide for slow release over an extended period. Such delivery

may decrease the dosage of drug required and may also decrease the number of treatments necessary to achieve a therapeutic effect.

The therapeutic oligopeptides of this invention may be prepared in accordance with conventional techniques, such as synthesis, recombinant techniques, or the like. For example, solid-phase peptide synthesis involves the successive addition of amino acids to create a linear peptide chain (see Merrifield (1963) J. Am. Chem. Soc. 85:2149-2154). Production of the peptide by recombinant DNA technology may also be performed. One first synthesizes or otherwise creates a nucleic acid sequence that encodes the desired peptide. This coding sequence is operably connected to suitable control elements for expression, e.g. promoters, terminators, ATG start codon, and the like as known in the art. In addition, DNA sequences encoding certain functional polypeptide elements such as signal sequences or proteins for targeting the peptide to specific intracellular compartments may be joined to the peptide within the expression cassette. This expression construct is introduced into a suitable host cell, and the recombinant protein that is produced is isolated. Alternatively, the coding sequence is introduced into the host to be treated for long term therapy, for example by inserting an expression construct into muscle or long-lived hematopoietic cells for therapy. The expression vector may be a plasmid, viral vector, including retrovirus, adenovirus, etc., and may be introduced by transduction, DNA vaccination, etc.

Pharmaceutically acceptable salts of the peptides also fall within the scope of the compounds as disclosed herein. The term "pharmaceutically acceptable salts" as used herein means an inorganic acid addition salt such as hydrochloride, sulfate, and phosphate, or an organic acid addition salt such as acetate, maleate, fumarate, tartrate, and citrate. Examples of pharmaceutically acceptable metal salts are alkali metal salts such as sodium salt and potassium salt, alkaline earth metal salts such as magnesium salt and calcium salt, aluminum salt, and zinc salt. Examples of pharmaceutically acceptable ammonium salts are ammonium salt and tetramethylammonium salt. Examples of pharmaceutically acceptable organic amine addition salts are salts with morpholine and piperidine. Examples of pharmaceutically acceptable amino acid addition salts are salts with lysine, glycine, and phenylalanine.

The subject methods are used to treat individuals suffering from demyelinating autoimmune disease. Diagnosis of suitable patients may utilize a variety of criteria known to those of skill in the art. A quantitative increase in myelin \square autoreactive T cells with the capacity to secrete IFN-gamma is associated with the pathogenesis of MS and EAE. During the pre-symptomatic period there is infiltration of leukocytes into the cerebrospinal fluid, inflammation and demyelination. Family histories and the presence of the HLA haplotype DRB1*1501, DQA1*0102, DQB1*0602 are indicative of a susceptibility to the disease. Treatment during the early stages of the disease is preferred, in order to slow down or arrest the further loss of neural function, although treatment at later stages of the disease is carried out to prevent or slow further progression of the disease.

Patients are diagnosed as having multiple sclerosis according to conventional clinical criteria. Such criteria rely on the presence of two attacks at least one month apart, where an attack is a sudden appearance of or worsening of an MS symptom or symptoms which lasts at least 24 hours; and more than one area of damage to central nervous system myelin. The damage to myelin must have occurred at more than one point in time and not have been caused by any other disease that can cause demyelination or similar neurologic symptoms.

MRI (magnetic resonance imaging) is the preferred method of imaging the brain to detect the presence of plaques or scarring caused by MS, although CT scans may also be used. Other symptoms include disability in mental, emotional, and language functions, movement and coordination, vision, balance, and the functions of the five senses. Evoked potential tests are electrical diagnostic studies which can show if there is a slowing of messages in the various parts of the brain, and may provide evidence of scarring along nerve pathways that is not apparent on a neurologic exam. Cerebrospinal fluid, usually taken by a spinal tap, may be tested for levels of cytokines, and for the presence of oligoclonal antibody band.

The therapeutic effect may be measured in terms of clinical outcome, or may rely on immunological or biochemical tests. Suppression of the deleterious T-cell activity can be measured by enumeration of myelin-reactive Th1 cells in spinal fluid, by quantitating the release of cytokines at the sites of lesions, or using other assays for the presence of autoimmune T cells known in the art.

Alternatively, one may look for a reduction in symptoms of a disease, such as the damage to neural tissue observed in MS, or the decrease in the number or severity of attacks of MS suffered by MS patients. Damage to neural tissue can be assessed for example by magnetic resonance imaging (MRI) and measurement of the number and severity of lesions visible therein. Reduction in MS attack number or severity can be assessed for example by clinical evaluation of patients. Methods for both MRI and clinical evaluation are well-known in the art.

Various methods for administration may be employed. The formulation may be given orally, by inhalation, or may be injected, e.g. intravascular, intra-tumor, subcutaneous, intraperitoneal, intramuscular, etc. The dosage of the therapeutic formulation will vary widely, depending upon the nature of the disease, the frequency of administration, the manner of administration, the clearance of the agent from the host, and the like. The initial dose may be larger, followed by smaller maintenance doses. The dose may be administered as infrequently as weekly or biweekly or monthly or on a schedule determined by the ordinarily skilled physician when administering a vaccine-like therapeutic, or fractionated into smaller doses and administered daily, semi-weekly, etc. to maintain an effective dosage level. In many cases, oral administration will require a higher dose than if administered intravenously.

The therapeutic ordered peptides of the invention can be incorporated into a variety of formulations for therapeutic administration. More particularly, the complexes can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, gels, microspheres, and aerosols. As such, administration of the peptides can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intracheal, etc., administration. The peptides may be systemic after administration or may be localized by the use of an implant that acts to retain the active dose at the site of implantation.

In pharmaceutical dosage forms, the peptides may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone

or in appropriate association, as well as in combination with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the therapeutic ordered peptides can be used alone
5 or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as
10 talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The therapeutic ordered peptides can be formulated into preparations for injections by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic
15 acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The peptides can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into
20 pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the therapeutic ordered peptides can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The therapeutic ordered peptides of the present invention
25 can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example,
30 teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition containing one or more compounds of the present invention. Similarly, unit dosage forms for injection or intravenous administration may comprise the compound of the present invention in a composition as a

solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

Implants for sustained release formulations are well-known in the art. Implants are formulated as microspheres, slabs, *etc.* with biodegradable or non-biodegradable polymers. For example, polymers of lactic acid and/or glycolic acid form an erodible polymer that is well-tolerated by the host. The implant containing therapeutic ordered peptides is placed in proximity to the site of action, so that the local concentration of active agent is increased relative to the rest of the body.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of peptides of the present invention calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the novel unit dosage forms of the present invention depend on the particular complex employed and the effect to be achieved, and the pharmacodynamics associated with each complex in the host.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

The compositions of the invention may also contain other therapeutically active agents, *e.g.* immunomodulators, immunosuppressants, β -interferon, steroids, statins *etc.* Of particular interest are combinations with other agents capable of additive or synergistic effect in achieving a therapeutic result, *e.g.* where a different or complementary pathway is affected by each of the active agents. Immunosuppressants of interest include cyclosporins A and G, FK-506, mycophenylate mofetil, rapamycin, azathioprine, antibodies for plasma membrane proteins associated with graft rejection, such as antibodies to CD4, CD8, CD2, LFA-1, ICAM-1, CD28, and the like; and immunosuppressive oligopeptides derived from MHC molecules. Antibacterial, antiviral and antifungal

drugs may also be co-formulated in order to minimize the effects of immunosuppression.

Depending on the patient and condition being treated and on the administration route, the therapeutic ordered peptides will generally be administered in dosages of 0.01 mg to 500 mg V/kg body weight per day, e.g. about 20 mg/day for an average person. The range is broad, since in general the efficacy of a therapeutic effect for different mammals varies widely with doses typically being 20, 30 or even 40 times smaller (per unit body weight) in man than in the rat. Similarly the mode of administration can have a large effect on dosage. Thus for example oral dosages in the rat may be ten times the injection dose. A typical dosage may be one injection daily.

Those of skill will readily appreciate that dose levels can vary as a function of the specific compound, the severity of the symptoms and the susceptibility of the subject to side effects. Some of the specific peptides are more potent than others. Preferred dosages for a given complex are readily determinable by those of skill in the art by a variety of means. A preferred means is to measure the physiological potency of a given compound.

It is to be understood that this invention is not limited to the particular methodology, protocols, formulations and reagents described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a complex" includes a plurality of such complexes and reference to "the formulation" includes reference to one or more formulations and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or

testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the methods and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the subject invention, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to ensure accuracy with respect to the numbers used (e.g. amounts, temperature, concentrations, etc.) but some experimental errors and deviations should be allowed for. Unless otherwise indicated, parts are parts by weight, molecular weight is average molecular weight, and pressure is at or near atmospheric.

20

EXPERIMENTAL

Example 1

Therapeutic Ordered Peptides for Immunomodulation Based on MHC-TCR Binding Motifs.

The region between the amino acids 85 to 99 of myelin basic protein (MBP) contain the immunodominant epitope for T cells and autoantibodies in MS brain lesions. The main region of MBP recognized by T cells and autoantibodies, found in MS brain, is the core motif, {SEQ ID NO:8} HFFK, from MBPp87-99 in patients who are HLA DRB1*1501 DQB1*0602 (HLA DR2).

Previously, we have compared the structural requirements for autoantibody recognition to those of T cell clones reactive to MBP p87-99. Anti-MBP antibodies were affinity-purified from CNS lesions of 12 post-mortem cases studied. The MBP p87-99 peptide was immunodominant in all cases and it inhibited autoantibody binding to MBP by more than 95%. Residues contributing to autoantibody binding were located in a 10-amino acid segment p86-95 ({SEQ

ID NO:9} VVHFFKNIVT) that also contained the MHC -T cell receptor contact residues for T cells recognizing MBP in the context of DRB1*1501 and DQB1*0602. In the epitope center, the same residues, {SEQ ID NO:10} VHFFK, were important for T cell binding and MHC recognition. Recently, the crystal
5 structure of HLA-DR2 with MBPp85-99 was solved, confirming the prediction that K91 is the major TCR contact residue, while F90 is a major anchor into the hydrophobic P4 pocket of the MHC molecule.

Peptides were synthesized that contained repetitive sequences of three amino acids ordered to bind the pockets existing in MS related MHC molecules and therefore to interfere with the activation of pathogenic T cells. One of those
10 predicted sequences ({SEQ ID NO:4} EYYKEYYKEYYK), was effective in preventing and treating experimental autoimmune encephalomyelitis in Lewis rats, an animal model of Multiple Sclerosis.

15 **Materials and methods.**

Animals. Female Lewis rats (6-8 weeks old), were purchased from Harlan Sprague Dawley (Indianapolis, IN)

Peptides. For immunization and disease reversal, peptides were
20 synthesized on a peptide synthesizer (model 9050: MilliGen, Burlington, MA) by standard 9-fluorenylmethoxycarbonyl chemistry. Peptides were purified by HPLC. Structure was confirmed by amino acid analysis and mass spectroscopy. Peptides used for the experiments were: {SEQ ID NO:11} ENPVVHFFKNIVTPR (MBPp85-99), {SEQ ID NO:4} EYYKEYYKEYYK, {SEQ ID NO:5}
25 KYYKYYKYYKYY.

EAE induction. Synthetic peptide MBPp85-99 was dissolved in PBS to a concentration of 2 mg/ml and emulsified with an equal volume of Incomplete Freund's Adjuvant (IFA), supplemented with 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). Rats were injected
30 subcutaneously with 0.1 ml of the peptide emulsion. Experimental animals were scored as follows: 0, no clinical disease; 1, tail weakness or paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb weakness or paralysis; 5, moribund or dead animal.

EAE treatment. Rats previously immunized with MBPp85-99 for EAE induction were scored from day eight after peptide injection. On the day of mean disease onset, animals were injected intraperitoneally with a solution of 0.5 mg of peptide in PBS (one dose of 0.25 ml).

Results.

Injection of ordered peptides containing TCR-MHC binding motifs reverse the development of EAE. In order to test the potential of the predicted sequences to revert the development of ongoing EAE we delivered a single dose of a PBS solution containing 0.5 mg of peptide in 0.25 ml. As seen in the graph (figure 1), this dose is enough to treat the ongoing disease, when compared with the control groups. In addition, ordered peptide treatment was administered in a mouse model of ongoing-EAE (Figure 2) at days 17, 29, and 36 after EAE induction with intravenous, intra-peritoneal or subcutaneous administration of EYYKEYYKEYYK (therapeutic ordered peptide) or Copaxone at two different dosages, 0.5 mg per mouse or 0.05 mg per mouse. Both the therapeutic ordered peptide and Copaxone were dissolved in mannitol. Results are expressed as relapse rates per mouse, and show that the therapeutic ordered peptide and Copaxone reduce relapse rates similarly.

Example 2

MHC Blockade and T Cell Antagonism of MBP Therapeutic Ordered Peptide in EAE

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A clear structural relationship between an autoimmune MHC-peptide complex and disease has not been demonstrated. Nevertheless, binding of peptides to the MHC has been extensively used as a parameter to select immunodominant sequences. Our results demonstrate a comparable amount of inhibition of binding of MBP peptide to rat MHC class II or PLP peptide to mouse MHC class II when either the therapeutic ordered peptide EYYK, the modified therapeutic ordered peptide, D-ala-EYYK, or glatiramer acetate were present in the reaction (Figure 3). These results show that the modified therapeutic ordered peptide D-

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Ala EYYK appears to have a higher affinity of binding to these MHC's than the unmodified EYYK, and approaches the binding achieved by Copaxone. Assays where inhibition of peptide-MHC binding by related structures is tested have been use to explain the mechanism of action of MHC blockade as a therapeutic tool.

- 5 By calculating the percentage of inhibition of MHC-PLP or MHC-MOG binding by the therapeutic ordered peptide the contribution of MHC blockade in the immunomodulatory effect of the ordered peptide is evaluated.

Peptide binding to class II molecules was measured as follows. Briefly, APCs (antigen presenting cells) were purified from spleen cells by negative selection
10 using magnetic beads (Dynal, Oslo, Norway) conjugated with antibodies specific for T cells (CD52), macrophages (CD45R), and NK cells (NKR-P1A) (Pharmingen, San Diego, CA). After selection, cells were plated at a concentration of 0.5×10^6 cells per well in flat bottom 96 well microtiter plates (Costar, Corning, NY). Therapeutic ordered peptides were used as inhibitors
15 are added to the wells at different concentrations (ranging from 0.01 to 0.24 mM) in a volume of 0.05 ml. After an hour of incubation at 37° C, 0.01 mM of biotinylated MBP, MOG, or PLP peptide was added. After four more hours of incubation, cells were harvested and binding of PE-Avidin to the cell surface was analyzed by FACS (Becton Dickinson, San Jose, CA). To determine the
20 IC₅₀ value of the therapeutic ordered peptide, for example EYYK, a linear regression curve was calculated using data points from 0.01mM to 0.80mM.

The ability of therapeutic ordered peptide EYYKEYYKEYYK, modified therapeutic ordered peptide D-Ala-EYYKEYYKEYYK-amide, or Copaxone to block the
25 proliferation of a PLPp139-151 specific T cell line was measured in a proliferation assay. As shown in the graph (figure 4) there is a dose dependent reduction in T cell proliferation with the modified therapeutic ordered peptide D-Ala EYYK that exceeds the reduction in proliferation with either the unmodified ordered peptide or Copaxone. This invention showed for the first time the modified therapeutic
30 ordered peptide D-ala EYYK was more effective in blocking T cell proliferation than the unmodified EYYK.

Example 3

5 Preparation of Modified MBP Therapeutic Ordered Peptide and Effect in EAE

The ability of therapeutic ordered peptide EYYKEYYKEYYK, modified therapeutic ordered peptide D-Ala-EYYKEYYKEYYK-amide, or Copaxone to treat a rat model of EAE was tested. A single 0.5 mg dose of each of these peptides was administered along with the encephalitogen (MBP 85-99 peptide in CFA) as an emulsion. As shown in Figure 5 the results, expressed as mean disease score of 12-13 animals, demonstrate surprisingly the superiority of the D-Ala form of the ordered peptide over the non-substituted form. The D-ala form nearly reaches the efficacy of Copaxone in this model.

15 Example 4

Preparation of MOG Therapeutic Ordered Peptide and Effect in EAE

In a similar fashion to example 3, the MOG therapeutic ordered peptide of, YREYEYE, either singly or in multimer form is prepared and tested in rat and mouse models of EAE. In addition a modified therapeutic ordered peptide D-alaYREYEYE is tested in both rat and mouse models of EAE.

Example 5

Preparation of PLP Therapeutic Ordered Peptide and Effect in EAE

25 In a similar fashion to example 3, the PLP therapeutic ordered peptide, YGKELGEY, either singly or in multimer form is prepared and tested in rat and mouse models of EAE. In addition a modified therapeutic ordered peptide D-ala YGKELGEY is tested in both rat and mouse models of
30 EAE.

Example 6

Administraion of MBP and MOG Therapeutic Ordered Peptides in Combination and Their Effect in EAE

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In a similar fashion to example 3, a combination of modified therapeutic ordered peptide D-Ala-EYYKEYYKEYYK-amide (the MBP therapeutic ordered peptide) and an the MOG therapeutic ordered peptide, YREYEYE, either singly or in multimer form and the D-ala modified form(s) are tested in rat and
10 mouse models of EAE. The combination is tested at various ratios of the two therapeutic ordered peptides as well as the two therapeutic ordered peptides fused into one larger peptide.

Example 7

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The Modified MBP Therapeutic Ordered Peptide Causes the Induction of Th2 Cytokines

One of the mechanisms proposed for the efficacy of Copaxone in MS is the induction of Th2 type of antigen-specific T cells that are beneficial in treating the
20 disease process. This mechanism was demonstrated in animals by the immunization of mice with both Copaxone and an encephalitogenic peptide, MBP 85-99, together in Incomplete Freund's Adjuvant (IFA) (Aharoni *et al.* (1997) Proceedings of the National Academy of Sciences USA 94:10821-10826). T cells were removed from the animals after approximately 10 days, grown in
25 culture for over 6 weeks, and then cytokine production by these Cop1 specific T cells were measured and determined to be of a Th2 type of cytokine pattern.

In a similar protocol we immunized mice with D-ala modified ordered peptide, non-modified ordered peptide, and control peptides including Cop1 and
30 ovalbumin (OVA). OVA is known to cause an induction of Th1 type of T cells after immunization. As shown in Figure 6, when cytokine production of individual T cells lines were measured from T cells stimulated with each of these antigens, there is an increased production of IL4 and IL10 in the D-ala-ordered peptide (D-

ala-EYYK) immunized T cell lines, but not in the non-modified ordered peptide (EYYK) immunized T cell lines. As controls, Cop1 caused an increase in these two Th2 cytokines as expected, and OVA did not cause an increase in these cytokines also as expected. These data imply that the D-ala modified form of the
5 ordered peptide can cause Th2 induction but that the unmodified peptide cannot.